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The Influence of Sucrose on Biomass and Glycosides Content of Callus Cultured from the Leaves of *Stevia rebaudiana* Bertoni

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Abstract

Stevia rebaudiana Bertoni contains diterpenoid steviol glycosides that have no adverse impact on blood sugar levels despite being 300 times sweeter than sugar. This study aimed to investigate the rate of callus induction from stevia leaves and the content of glycosides when changing the sucrose percentage in the culture medium.. Murashige and Skoog (MS) culture medium supported by 4.0 mg/l naphthalene acetic acid (NAA) and 1.0 mg/l benzyl adenine (BA) was used, and different concentrations of sucrose (2, 3, 4, 5 and 6%) were tested .The extraction of glycosides from leaf and callus tissues was performed by using methanol. Extracted glycosides were analyzed by high-performance liquid chromatography (HPLC). The results showed significant influences of sucrose on callus initiation. The concentration of 3% sucrose had the highest fresh and dry weight. No callus was induced in the MS medium with a high concentration of sucrose (5% and 6%). However, the highest glycoside content, stevioside and rebaudioside were obtained from callus treated with 4% concentration followed by 3% sucrose the treatment . The highest fresh and dry weight average was obtained with a 3% concentration of sucrose, this treatment also increased the concentration of glycosides in the callus twice as much as in the leaves, and concentrations of 3% and 4% of sucrose gave very similar concentrations of glycosides in callus in terms of being double what was found in the leaves something which may aid in the development of a stevia glycosides based industry.

Keywords: HPLC analysis, Sucrose concentrations, Callus induction, Stevia plant

تأثير السكروز على محتوى الكتلة الحيوية والكلايكوسيدات في الكالس المستزرع من أوراق نبات الستيفيا

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الخلاصة

يحتوي *Stevia rebaudiana* Bertoni على جليكوسيدات الستيفيول ثنائية التربينويد التي ليس لها تأثير سلبي على مستويات السكر في الدم على الرغم من كونها أحلى 300 مرة من السكر. هدفت هذه الدراسة إلى معرفة معدل تكوين الكالس من أوراق الستيفيا ومحتوى الكلايكوسيدات عند تغيير نسبة السكر في وسط الاستزراع. تم استخدام وسط استنبات مدعومًا بـ 4.0 مجم / لتر من حمض الخليك النفثالين و 1.0 مجم / لتر بنز الادنين

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، وتم اختبار السكروز (2 ،3 ، 4 ، 5, 6٪) بواسطة استخلاص الكلايكوسيدات من أنسجة الأوراق والكالس باستخدام الميثانول. تم تحليل الكلايكوسيدات المستخرجة بواسطة كروماتوغرافيا السائل عالية الدقة HPLC. أظهرت النتائج وجود تأثير معنوي للسكر على تكوين الكالس. كان لتركيز 3٪ سكر أعلى وزن من الأوراق الطازجة والجافة. ومع ذلك ، تم الحصول على المحتوى الكلايكوسيدات الساتيفوسيد والريبوديوسيد من الكالس المعالج بتركيز 4٪ ، متبوعًا بمعالجة 3 ٪ سكر. وجد انه عند التركيز 3٪ من السكر تم الحصول على أعلى متوسط للوزن الطازج والجاف بالاضافة الى زيادة تركيز الكلايكوسيدات في الكالس ومضاعفة تركيزه بمقدار الضعف عما في الأوراق ، وأعطت التركيزات 3٪ و 4٪ من السكر تركيزات متشابهة جدًا من الكلايكوسيدات في الكالس حيث لمضاعفة تركيزه عما في اوراق النبات الكامل،الامر الذي قد يساعد في تطوير صناعة تعتمد على كلايكوسيدات الستيفيا.

Introduction

Stevia rebaudiana (Bertoni) is a perennial herbaceous plant of the family Asteraceae that is also known as sweet leaf, honey leaf, and candy leaf. Stevia is native to Paraguay (South America) and Brazil[1]. Stevia comprises 280 species that reach a height of 1 m and have leaves measuring 2 to 5 centimeters in length. Stevia leaves are a source of binary turbine glycosides, including steviolbioside rubsoside, glucoside, and stevioside rebaudioside F, E, D, C, B, and A. The greater the amount of rebaudioside-A, the higher the quality[2].

Diterpene glycosides derived from stevia leaves are 300 times sweeter than sucrose and may thus be used as a replacement for sucrose[3]. Stevia is used to manufacture non-caloric sweeteners that are natural alternatives to artificial sweeteners. Since the human body does not metabolize them, stevia is considered safe for diabetics[4]. With the present need for low-carbohydrate, low-calorie, and low-sugar dietary supplements, the stevia plant and its extracts have shown to be the best option. The Food and Drug Administration's (FDA) approval of stevia as a dietary supplement demonstrates the herb's health benefits. In addition to its non-caloric sweetening qualities, *S. rebaudiana* has other essential therapeutic benefits. It treats various illnesses, including cancer, obesity, tooth decay, hypertension, weariness, and depression. It is hypoglycemic, hypotensive, vasodilative, taste-enhancing, sweetening, anti-fungal, anti-viral, anti-inflammatory, and anti-bacterial, and boosts the body's urine function. It is non-toxic, non-addictive, non-carcinogenic, non-mutagenic, non-teratogenic, and devoid of genotoxic properties. It does not affect blood sugar and is thus safe for people with diabetes. Consequently, it has considerable industrial and medicinal utility[5, 6].

Since stevia is incompatible with itself, a seed from a single plant would constitute a half-sibling family. Stevia is a short-day plant that blooms in the Southern Hemisphere from January to March and in the Northern Hemisphere from September to December. Fertile seeds are typically black, whereas infertile seeds are often light or transparent[7]. Stevia seeds have a meager germination rate and vegetative by cuts is constrained by the tiny population size[8]. Hence, tissue culture is the only fast approach for mass production of stevia. In order to produce whole plant from the separated meristem, to induce callus, and to form full plantlets via organogenesis or embryogenesis, cells are now cultivated in vitro in bulk or as clones from single cells [9].Tissue culuture is turning to be an alternative vegetative propagation approach of plant [10]. The fact that the most significant levels of stevioside and rebaudioside are discovered in the leaves implies that they are the principal tissue for stevioside production and accumulation [11]. The method of inducing and multiplying callus was developed by P.Gupta *et a*l. [12]. Explants of nodes, leaves, and roots are grown in Murashige and Skoog (MS) medium supplemented with various concentrations of benzyl adenine (BA) and naphthalene acetic acid (NAA). Callus development is more successful when initiated from leaf explants .

The production of secondary metabolites is also influenced by medium culture components such as nutrients, carbohydrates (sugars), growth regulators, and amino acids [13]. The prime carbon source in culture media is sucrose, which promotes cell growth, increases callus biomass, and aids molecular signals in activating the genes that code for the secondary metabolite biosynthetic enzymes [14]. Thus, the current study aimed to evaluate different sucrose concentrations effects on callus production (biomass) and determine the optimum concentration to enhance glycoside production from the callus.

Materials and Methods

Preparation of Explant

S. rebaudiana plants were obtained from a commercial company (Janet Al Nakheel for Plant Tissue Culture Co Ltd, Iraq) and identified by Dr. Sukayna Abbas Aliwy, the botanist in the herbarium of the Department of Biology, College of Science, University of Baghdad. Young and actively growing leaves were used as plant material. Plant leaves were rinsed gently with tab water. To sterilize the explant, 70% ethanol was used for 30 seconds, and then submerged in 2% sodium hydrochloride (after the addition of three drops of tween 20 to increase the efficiency of sterilization) for 5 min with continuous shaking and then rinsed three times with sterilized distilled water for 5 minutes, each time to remove any remaining clorox.

Callus Induction

Callus induction was performed using MS medium supplemented by 4.0 mg/l NAA and 1.0 mg/l BA, with sucrose in different concentrations of 2, 3, 4, 5 and 6% (10 replication per treatment). The media were solidified using five g/l agar-agar and sterilized in the autoclave for 17min at 121ºC under 1 bar of pressure after adjusting the media's pH to 5.8. Data was recorded as fresh and dry callus weight after two months of culture [15]. The experiment was repeated twice.

Extraction of Steviol Glycosides

Steviol glycoside extraction was done from *S. rebaudiana* leaves of the entire plant. Its callus culture was carried out following the method laid by Rashid *et al*. [16]with some modifications, 1g of the fresh weight of the callus. Mature leaves (considered as a control in estimating the glycosides) were added to 10 ml of 80% boiled methanol at 80ºC and pulverized using mortar. Each sample was completed to 50 ml methanol and then placed in water bath at 80ºC for 20 minutes. Next, samples were placed in centrifuged for 10 minutes at 6000. Finally, the supernatant was separated, poured into Petri dishes, and left in the oven at 40ºC for drying for 24 hours.

HPLC Analysis:

Qualitative and quantitative estimation of stevioside and rebaudioside in the extracts was performed by using HPLC analysis [17] under the following conditions of separation: Column: type C-18, 3µm size of particle 5mm, 250mm length, 4.6mm internal meter, mobile phase, deionized water acetonitrile (66:34%) isocratic**,** UV light of 200 nm was used for the detection, the volume of the injection was 20 µl**,** the flow rate was: 1 ml/minute and temperature at 25 ºC. These concentrations were calculated as follows: Concentration of compound (µg/g) $= \frac{\text{Peak area of compount}}{\text{Peak area of standard}} \times$ Concentration of standard × Dilution factor [18].

The samples were identified by measuring the retention time. Standards of stevioside and rebaudioside were used.

Statistical Analysis

Statistical Analysis System (SAS), version 2018 was utilized, to analyze the impact of independent variables on predefined metrics for this study. In this study, means were compared using the Least Significant Difference (LSD) test (Analysis of Variance-ANOVA) a probability value of 0.05 [19].

Results and Discussion

Effect of Different Concentrations of Sucrose on Callus Induction

A growing medium containing growth regulators and sugar was used to promote callus development. In the dark 4.0 mg/l NAA and 1.0 mg/l BA and 1.0 mg/l sucrose were added to MS media before inoculating with leaf explants. After four weeks, the induced callus began to expand. Following eight weeks of culture, the results of altering the sucrose content ara reported in Table 1.Sucrose concentrations significantly influenced calli's fresh and dry weight ($p \leq 0.05$). The results showed that the new and dry weight of callus increased from 2088 mg and 200.91 mg to 3395 mg and 395.45 mg at sucrose concentrations of 2% and 3%, respectively, and then decreased to 1175 mg fresh weight and 110 mg dry weight at a concentration 4% of sucrose. While high concentrations of sucrose (5% and 6%) depressed the callus formation. These results revealed that sucrose at 3% induced the most significant callus formation. Overall, the color of calli obtained from medium containing 4.0 mg/l NAA and 1.0 mg/l BA and different sucrose concentrations (2,3 and 4%) had the same color: light yellow and the calli texture was crispy in all treatments (Figure 1).

Table 1: Fresh and dry weight of stevia callus using different concentrations of sucrose induced on MS-supported culture medium with 4.0 mg NAA and 1.0 mg BA (n=10)

Concentration of Sucrose (%)	Fresh Weight (mg)	Dry Weight (mg)
2	2088.00	200.91
3	3395.00	395.45
4	1175.00	110.00
5	----	----
6	----	----
LSD value	102.47*	26.44*

*($P \leq 0.05$)

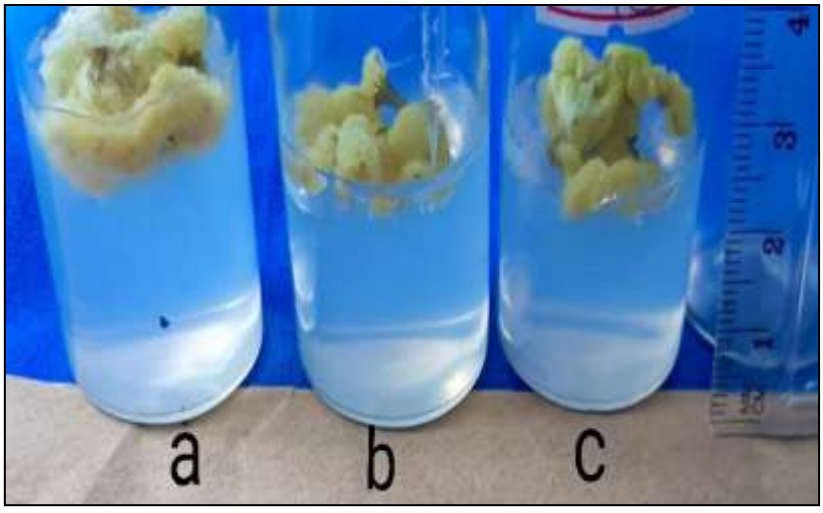


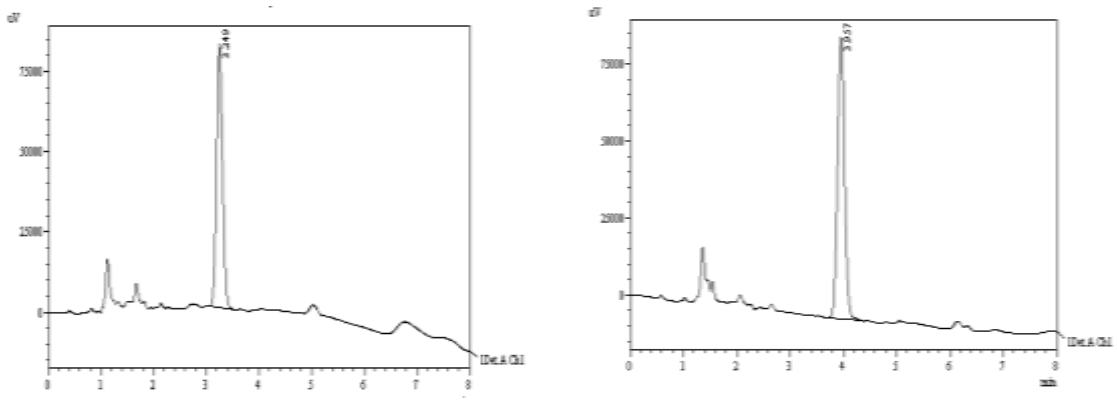
Figure 1: *In vitro* callus induction from leaf explants of *S. rebaudiana* on MS medium supported by 4.0mg/l NAA , 1.0mg/l BA and different concentrations of sucrose. a: Treatment with 3% of sucrose. b: Treatment with 2% of sucrose. c: Treatment with 4% of sucrose.

The results proved that the sucrose concentration in the culture media had an apparent effect on the induction of callus from *S. rebaudiana* leaves. The maximum plant fresh and dry weight was observed at 3% concentration of sucrose and the minimum plant fresh and dry weight was shown at 4% concentration of sucrose, while each of 5% and 6% concentrations did not affect callus induction[20] which may be due to the increase in the amount of sucrose in the culture medium[21], or due to the rise of sucrose induced osmotic stress resulted in a reduction in the relative growth rates. Stress-induced with 5% and 6% sucrose resulted in callus death (negative relative growth rate) [22]. These findings were consistent with those of [23], who found that a concentration of 3% sucrose resulted in the best callus development and may, therefore, be used for future use in mass manufacturing. Samuel *et al*. [24] also examined different carbohydrates effects on callus proliferation in pineapple and reported that the highest amount of callus dry matter was obtained in MS medium with 3% sucrose. Sucrose was the best carbon source for callus proliferation.

HPLC Analysis

Identification of steviol glycosides, stevioside, and rebaudioside was carried out from the extracts of calli derived from *in vitro* culture and were then compared with the content of extract of intact plant leaves (control). Standard stevioside and rebaudioside were used for identification. The glycoside content was identified in the samples by comparing the retention time and peak area of the sample with that of the standard. As a result, rebaudioside appeared at 3.249 minutes retention time with a 667135 µvolt peak area (Figure 2:a), while stevioside appeared at 3.922 minutes retention time with a 842628 µvolt peak area (Figure 2:b).

HPLC analysis of *Stevia rebaudiana* fresh leaves (control) showed that rebaudioside was present at 3.214 minutes retention with a 151881µvolt peak area of 151881µvolt, while the occurrence of stevioside was at 3.933 minutes retention time with a 9592 µvolt peak area (Figure 2:c). Rebaudioside and stevioside appeared at 3.248 and 4.044 minutes retention times with 591417 and 221066 µvolet peak areas respectively, in *Stevia rebaudiana* callus extract from 2% sucrose treatment (Figure 2: d). There was appearance of rebaudioside and stevioside at 3.250 and 4.029 minutes a retention times with 730868 and 235478 µvolet peak areas respectively, in *Stevia rebaudiana* callus extract from 3% sucrose treatment (Figure 2: e). Rebaudioside and stevioside appeared at retention times of 3.254 and 4.019 minutes with a peak areas of 748337 and 244638 µvolet, respectively, in *Stevia rebaudiana* callus extract after 4% sucrose treatment (Figure 2: f).



(a) **(b)**

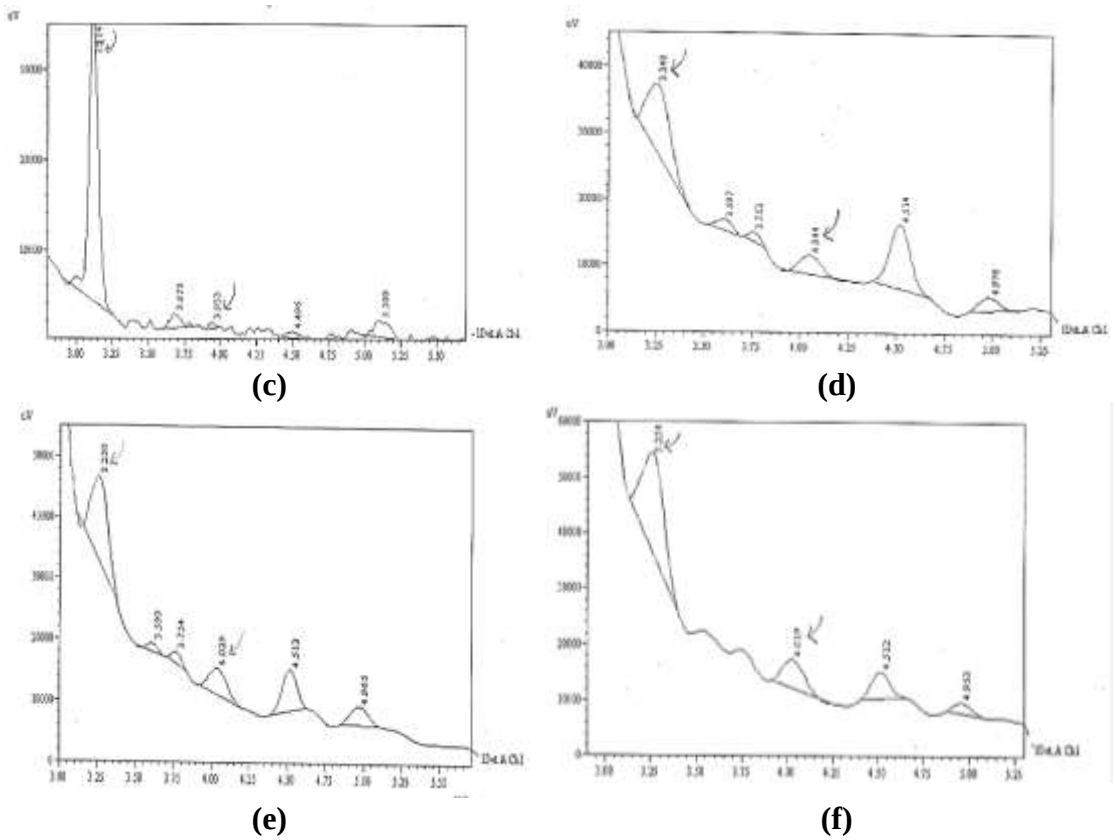


Figure 2: HPLC analysis of glycosides of *S. rebaudiana*. a. Rebaudioside standard. b. Stevioside standard. c. Extract intact plant leaves. d. Extract of callus at 2% of sucrose. e. Extract of callus at 3% of sucrose. f. Extract of callus at 4% of sucrose**.**

The content of rebaudioside and stevioside in the extracts of all tested parts is presented in Table 2. Results showed that the highest concentration of rebaudioside was 112 µg/g and stevioside 29µg/g at 4% concentration of sucrose, followed by 109.5 µg/g of rebaudioside and 27 µg/g of stevioside at 3% sucrose.

Table 2: Effect of different concentrations of sucrose in MS medium with 4.0 mg/l NAA and 1.0 mg/l BA on rebaudioside and stevioside quantity (µg/g) after eight weeks of culture.

	Rebaudioside			**Stevioside**		
Sample	**Retention Time (min)**	**Peak Area (µvolet)**	**Concentration (µg/g)**	**Retention Time (min)**	**Peak Area (µvolet)**	**Concentration (µg/g)**
Intact plant leaves	3.214	15188	45.5	3.933	9592	2.27
Sucrose 2%	3.248	591417	88.6	4.044	221066	26.2
Sucrose 3%	3.250	730868	109.5	4.029	235478	27
Sucrose 4%	3.254	748337	112	4.019	244638	29

The results of HPLC, shown in Table 2, revealed that the concentration of glycosides (stevioside and rebaudioside) in callus increased with sucrose in the culture medium. The highest concentration of both was obtained at 4%. This increase in the concentrations may be

due to exposure of plant cells to stress, in comparison with stable conditions, which contributeds to the rise in the accumulation of secondary metabolites in callus tissues, but not under excessive stress of the cells as in 5% and 6% sucrose concentrations [25]. Additionally, the media in tissue culture provide the requirements from nutrients, regulators, and other additions for cell division and the best productivity of secondary metabolism [26]. Therefore, an optimal medium must be provided to increase the biomass of cells and their bioactive compounds.

Conclusions

1- The highest average of fresh and dry weight was obtained at 3% sucrose concentration.
2- A higher concentration (5 and 6%) of sucrose depressed callus induction.
3- 3% and 4% of sucrose gave approximately similar concentrations of glycosides in the callus.
4- The content of glycosides in the callus was more than two times its concentration in the leaves of the entire plant, and this finding may help establish a stevia glycosides-based industry

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